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# Occurrence in the Field of a Long-Term, Year-Round, Stable Population of Placozoans

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**Abstract.** Long-term field studies on placozoans (*Trichoplax adhaerens*), including both substrate sampling and slide sampling, were carried out at a subtidal site near Shirahama, Japan. Samples of natural substrate materials from the field, such as stones, shells, or fragments of coral, were particularly useful for obtaining placozoans. Results from the substrate sampling indicate that placozoans are present year-round at the study site. Large intermittent peaks in the number of animals collected at the study site occurred roughly once a year, between late summer and the beginning of winter. Placozoans were present every year from 1989 through 2000. A seawater aquarium was also studied and provided a considerable number of placozoans for more than 1 year.

## Introduction

The placozoan *Trichoplax adhaerens* F. E. Schulze is a ciliated microscopic marine animal with a platelike morphology (Schulze, 1883; Grell, 1971, 1982; Miller, 1971a, b; Pearse *et al.*, 1987; Margulis and Schwartz, 1988; Grell and Ruthmann, 1991; Conn, 2000; Brusca and Brusca, 2003). The thin body consists only of an epithelium and an internal mesenchyme. The epithelium has two regions: an upper free, or dorsal, epithelium of cover cells and a lower attached, or ventral, epithelium of cylinder and gland cells (Grell and Benwitz, 1971; Pearse *et al.*, 1987; Grell and Ruthmann, 1991). Some differences between the central and marginal areas in the body have also been reported (Schwartz, 1984; Schuchert, 1993; Pearse *et al.*, 1994). When the densely ciliated ventral epithelium is in contact with the substrate, the animals display a gliding (or creeping) locomotion (Grell and Ruthmann, 1991). When the animal is fed, its shape changes periodically (Ueda *et al.*, 1999). For example, in addition to the gliding amoeboid platelike form, other forms (swarmers) (Thiemann and Ruthmann, 1991) have been described. Further, a swimming form has been reported (cited in Margulis and Schwartz, 1988; also see Levin and Bridges, 1995). *Trichoplax adhaerens* is considered to be the sole species in the phylum Placozoa (Grell, 1982; Grell and Ruthmann, 1991; Brusca and Brusca, 2003).

Placozoans have been found in seawater aquaria (Schulze, 1883; Miller, 1971a, b; Pearse *et al.*, 1987; Margulis and Schwartz, 1988; Grell and Ruthmann, 1991), as well as in various warm coastal areas (Grell and Benwitz, 1971; Sudzuki, 1977; Grell and López-Ochoterena, 1987; Pearse, 1989; Uehara *et al.*, 1989; Pearse *et al.*, 1994; V. B. Pearse, pers. comm.). Nevertheless, the biology of this microscopic animal under natural conditions is little known (Grell and Ruthmann, 1991).

In this study, which is based on long-term sampling of both glass slides and natural substrates, the occurrence of placozoans was examined at a subtidal site as well as in a seawater aquarium at the Seto Marine Biological Laboratory in Shirahama, Japan. Results of this study indicate that, in addition to their abundance in the seawater aquarium, placozoans are present at the study site year-round.

## Materials and Methods

### *Sampling in the field*

The study site was located at the east side of Tanjiriku-zurenohana on the southern coast of Tanabe Bay, near the

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Seto Marine Biological Laboratory of Kyoto University at Shirahama (Wakayama Prefecture, Japan). It was situated in the upper portion of the subtidal zone, about 1 m below the datum line in the tide table for Shirahama (tide tables issued by the Japan Meteorological Agency, Tokyo). Water temperatures were recorded (Fig. 1).

*Slide sampling.* Following Pearse (1989), specimens of placozoans were collected on glass slides (76 mm × 26 mm). Typically, seven clean slides were placed in a stainless steel slide rack (72 mm × 66 mm × 31 mm; ordinarily used in histology), and four sets of racks thus prepared were placed on the substrate at the study site. Later (19 to 68 days; 35 days on average), each of the racks was put separately into a plastic container filled with ambient seawater collected at the site and returned to the laboratory for observation. Sometimes, individual clean slides, not in racks, were placed at the site, and individual slides were also examined if the racks holding them disappeared unaccountably. On the day of retrieval, or the day after, new sets of slides were placed for the next observation.

*Substrate sampling.* Placozoans were also collected from samples of natural substrate materials ranging in size from pebble to cobble (according to the particle grade scale of Wentworth; *e.g.,* Lincoln *et al.,* 1998); for example, those of 4–8 cm in length are within this range. Stones, shells of molluscs (dead or alive), fragments of skeleton of hard corals (dead or alive), or mixtures thereof, were collected by hand at the study site and placed separately into containers of ambient seawater. These samples were brought to the laboratory for observation. The sampling was carried out at intervals of 12–68 days, 35 days on average.

*Laboratory observations of placozoans from the field.* In the slide sampling, each slide was quickly transferred into a plastic dish (90 mm in diameter) containing seawater from the field. Both surfaces of each slide were examined under a dissecting microscope. In the substrate sampling, each sample container was typically treated as follows. It was shaken for several seconds, and the resultant suspension was decanted into plastic dishes (90 mm in diameter). A few hours later, these dishes were observed under a dissecting microscope for placozoans, and within a few days (mostly, the day after), they were observed again. Placozoans could be seen; they were attached to and gliding along the bottom of the plastic dishes.

When placozoans were found in these samples or on slides from the field (Fig. 2), they were transferred, with a mouth-controlled micropipette, to an observation chamber and further observed with a light microscope equipped with Nomarski optics. The thin platelike body form attached to the substrate on its ventral surface, its gliding ciliary movement, and the presence of shining spheres in the dorsal epithelium served to identify these animals as placozoans (Grell and Benwitz, 1971; Miller, 1971a; Grell and Ruthmann, 1991). Furthermore, the occurrence of birefringent granules in the subperipheral region (Miller, 1971a; Pearse *et al.,* 1994) usually served for identification.

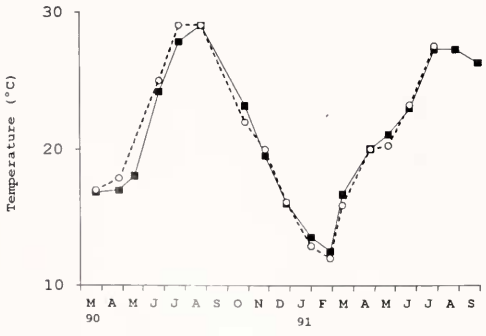


**Figure 1.** A profile of changes in water temperature at the study site and in the seawater aquarium. Abscissa: months from March 1990 through September 1991. Ordinate, temperature (°C) for the study site (squares, ■) and for the seawater aquarium (circles, ○).

### *Measurement of size in placozoans*

Because the shape of placozoans continuously changes, determinations of size are only valid at the moment of measurement. Furthermore, only placozoans that were attached to either a glass or plastic substrate were measured. Under those circumstances, the lengths along the longest and shortest axes were measured with an ocular scale under a dissecting microscope, and the average of the two values was taken as the size of the individual.

### *Source of placozoans collected from stones*

Samples of stones were collected at the study site and another subtidal site in Tanabe Bay (see a later section) and transported to the laboratory. Each sample consisted of a stone (4–8 cm in length) and an aliquot (typically, 30–70 ml) of the ambient seawater in a container. The stone in the container was transferred by hand into an aliquot (typically, 30–40 ml) of artificial seawater (ASW; Jamarin U, Jamarin Laboratory, Osaka) in a separate container; the ambient seawater (SW) was retained. Both containers—one with the ASW and the stone, and the other with the retained SW—were shaken similarly, and the fluids were then examined individually for placozoans, as described above; meanwhile the stone was discarded. The relative difference in the proportion of placozoans in the ASW and in the SW was calculated according to the expression $[(A - B)/(A + B)] \times 100$, where $A$ is the number of placozoans in the ASW, and $B$ is the number in the SW. The differences were

**Figure 2.** A placozoan on a slide from the study site. Bar, 100 μm.

statistically tested with a Student's *t* test, after arcsine transformation (Sokal and Rohlf, 1995) (Table 1).

*Sampling at a seawater aquarium*

Placozoans were also collected from the Kyoto University Aquarium at the Seto Marine Biological Laboratory. In a seawater aquarium in this facility, a square plastic basket was suspended; many hard corals were reared in the basket, and running seawater was continuously supplied from above. Water temperatures were recorded (Fig. 1).

Individual clean glass slides (76 mm × 26 mm), not in racks, were used here for sampling placozoans (slide sampling). About 10 slides were placed on the bottom of the basket with the corals. Later (19 to 44 days, 31 days on average), the slides were transferred to a container filled with seawater taken from the seawater supply system and were returned to the laboratory for observation under a dissecting microscope, as described above. When slides were removed, a new set of clean slides was placed for the next observation.

## Results

*Slide sampling at the subtidal study site*

Slide samplings at the study site were carried out at intervals of 1 month on average for about 3 years, from November 1989 through December 1992. The samples yielded a total of 230 placozoans, which appeared in 13 of 33 (39%) samplings on 63 of 818 (7.7%) retrieved slides.

The number of placozoans changed with time, with large intermittent peaks in November 1990, September 1991, and August 1992 (arrows in Fig. 3). The percentage of slides with placozoans (placozoan-positive slides, or positive slides) was also higher in these months (Fig. 3). In addition, the number of racks containing slides with placozoans was also high in these months: 4 out of 4 in November 1990, 4 out of 4 in September 1991, and 3 out of 4 in August 1992.

The placozoans ranged in size from about 100 μm to 1200 μm (394 ± 179 μm, the mean ± SD of 227 individuals). All exhibited the platelike morphology (Fig. 2); but two placozoans obtained in November 1990 had a transparent balloonlike protrusion on the dorsal side, and two placozoans collected in September 1991 had a small protrusion.

*Substrate sampling at the study site*

Placozoans were found on all kinds of the substrate materials tested (stones, molluscan shells, corals), suggesting no specificity of placozoans for these particular substrates.

When samples of these materials were examined after collection at intervals of about 1 month (12–68 days, 35 days on average), for 2½ years, from July 1990 through December 1992, placozoans were found on all but two occasions (92%, 24 out of 26) (Fig. 4). Thus, placozoans were present virtually throughout the year at the study site.

A total of 199 placozoans were obtained from 39 out of the 114 (34%) samples. A large number of placozoans were obtained in November 1990 and August 1992 (Fig. 4). The percentage of positive samples varied with time, with peaks

**Table 1**

*Source of placozoans collected from field samples of stones and ambient seawater*

| | Numbers of placozoans[a] | | |
|---|---|---|---|
| Experiments | ASW + stone | SW | % Difference[b,c] |
| 1 | 6 | 0 | 100 |
| 2 | 5 | 2 | 43 |
| 3 | 2 | 0 | 100 |
| 4 | 1 | 0 | 100 |
| 5 | 1 | 0 | 100 |

[a] The stone from each sample was transferred to artificial seawater (ASW), the stone and ASW were shaken, and the ASW was then examined for placozoans, as was the retained ambient seawater (SW) (see protocol in Materials and Methods). Only results from placozoan-positive samples are shown.

[b] % Difference, the relative value of the difference in number was calculated according to the equation $[(A - B)/(A + B)] \times 100$, where $A$ and $B$ represent the number of placozoans from ASW (+ stone) and SW, respectively.

[c] The % difference is significantly greater than 0% ($P < 0.05$); *t* test (see Materials and Methods).

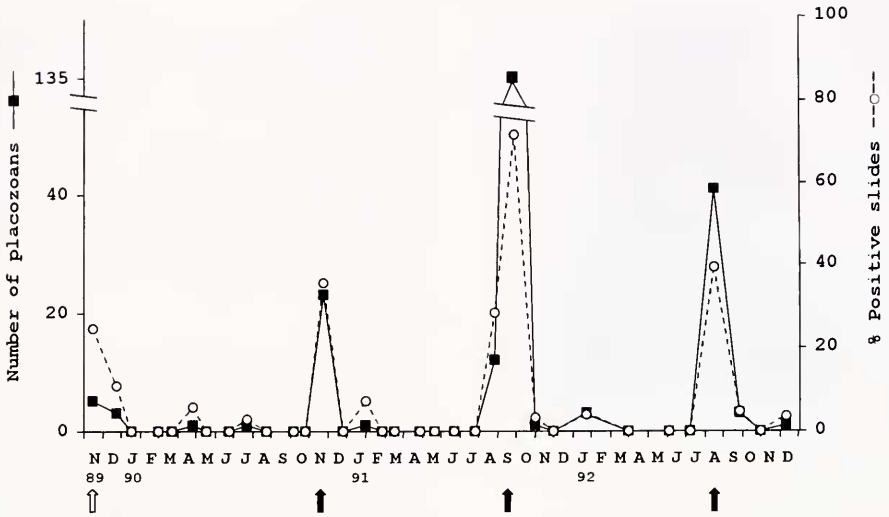


**Figure 3.** Slide sampling at the study site. Abscissa, months from November 1989 through December 1992. Ordinate (left), number of placozoans obtained from slides at each sampling (squares, ■); ordinate (right), percentage of placozoan-positive slides (circles, ○). Slides (10–34, 25 on average) that had been placed at the study site for about 1 month (19–68 days, 35 days on average) were used for the observations. Filled arrows indicate the timing of large intermittent peaks (November 1990, September 1991, and August 1992). The peak in November 1989 was also probably in this class (open arrow).

appearing in October 1990 to January 1991, September 1991, and August 1992 (see Fig. 4).

The placozoans obtained from substrate materials were, however, small. The animals collected from some samples ranged from 100 $\mu$m to 400 $\mu$m (132 $\pm$ 77 $\mu$m, mean $\pm$ SD of 52 individuals), and were frequently damaged at the periphery. Thus many of these placozoans had probably been fragmented during the sampling procedure. All, however, exhibited a platelike morphology.

*Field sampling from 1993 through 2000*

More recently, (August 1993–November 2000), samplings for placozoans, on either slides or natural substrates, were continued at the study site at a frequency of 1–3 times per year. Placozoans were present in every year. Moreover, placozoans were also present at other sites in this area of Shirahama, including a subtidal site and a tidal pool in Tanabe Bay as well as in Kanayama Bay.

*Source of the placozoans collected from field samples of stones*

Experiments were carried out to determine whether the placozoans obtained by substrate sampling were associated with the substrate or with the ambient seawater. Field samples of stones in containers of ambient seawater were used for this purpose ($n$ = 19). The stone from each sample was individually transferred to artificial seawater (ASW + stone), the combination was shaken, and the ASW and the retained ambient seawater (SW) were examined for placozoans. Only two placozoans (mean size 94 $\mu$m) were found in the SW from 1 out of 19 samples, but 15 placozoans (85 $\pm$ 28 $\mu$m) were collected in the ASW from 5 out of 19 samples.

As shown in Table 1, in all these positive samples, placozoans were obtained from the ASW, and the placozoans were not equally distributed in the ASW and the SW (15 in the ASW and 2 in the SW; $P < 0.05$); and the proportion of placozoans in the ASW was enriched compared with that in the SW (Table 1). An association of some placozoans with the substrate (or other substrate material on it) rather than with the ambient seawater was suggested.

*Sampling in the seawater aquarium*

In earlier years, from November 1989 through August 1991, a seawater aquarium was also examined for placozoans. The slide sampling, carried out at intervals of 1 month

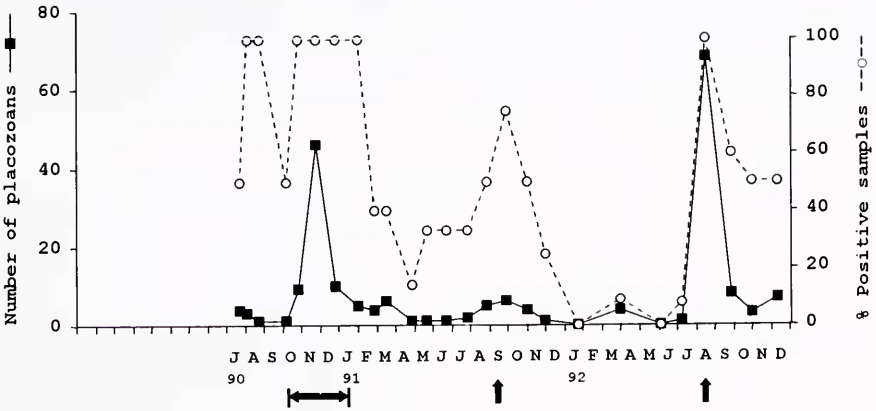


**Figure 4.** Substrate sampling at the study site. Abscissa, months from July 1990 through December 1992. Ordinate (left), number of placozoans obtained from samples of substrates at each sampling (squares, ■); ordinate (right), percentage of placozoan-positive samples (circles, ○). Samples of natural substrates were collected at intervals of about 1 month (12–68 days, 35 days on average). The number of samples was variable (1–13, 4 on average); 1–2 from July 1990 through January 1991, 3–7 from February 1991 through December 1991, 7–13 from January 1992 through July 1992, and 3–6 from August 1992 through December 1992. Arrows indicate peaks (October 1990 to January 1991, September 1991, and August 1992). The timing of the peaks is matched with that of the large intermittent peaks in Fig. 3.

on average for 1 ½ years, yielded a total of 1144 placozoans from the seawater aquarium (Fig. 5). The animals were obtained on most occasions (19 out of 22, or 86%) and inhabited 98 of 218 (45%) retrieved slides.

Both the number of placozoans at each sampling and the percentage of slides with placozoans (positive slides) varied with time (Fig. 5). Many placozoans were obtained in January, March, and April 1990, and August 1991. The percentage of positive slides was also high in these months.

All of the placozoans exhibited a platelike morphology. In one sampling, they ranged in size from about 100 $\mu$m to 300 $\mu$m (257 ± 59 $\mu$m, the mean ± SD of 8 individuals).

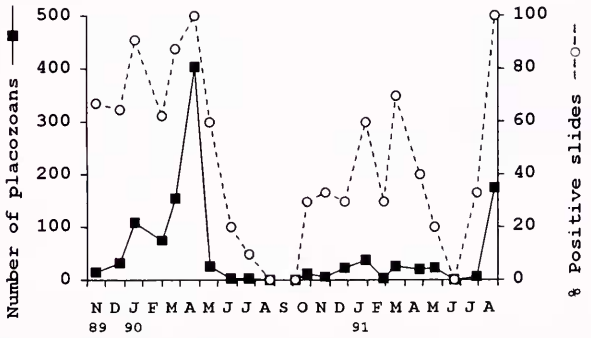


**Figure 5.** Slide sampling in the seawater aquarium. Abscissa, months from November 1989 through August 1991. Ordinate (left), number of placozoans obtained from slides at each sampling (squares, ■); ordinate (right), percentage of placozoan-positive slides (circles, ○). Slides (3–15, 10 on average) that had been placed in the seawater aquarium for about 1 month (19–44 days, 31 days on average) were observed. Note that the scale of the left ordinate is much larger than those in Figs. 3 and 4.

## Discussion

This study, carried out from November 1989 to November 2000, at a study site in the subtidal waters in Shirahama, showed that placozoans were present year-round and in every year. Moreover, placozoans were also observed in July 1989 at and near To Island in Tanabe Bay (V. B. Pearse, pers. comm.). Further, a seawater aquarium used to hold transient stocks of local marine animals also yielded, over 1 year, a large number of placozoans. Thus, these animals have been observed for more than 11 years in the Shirahama area, and therefore, the population of placozoans can be considered to be stable.

Unexpectedly, the number of placozoans collected at the subtidal study site showed large, intermittent peaks that occurred roughly once a year, between late summer and the beginning of winter. Although the habitat of the seawater aquarium was quite different from that in the field, a peak also appeared in the aquarium samples. But this peak was shifted from that observed in the field samples. The significance of these fluctuations is unresolved.

Substrate sampling in this study was more direct and more productive for obtaining placozoans in the field than the slide sampling. But the number of organisms collected from substrate material is not certain, because some placozoans are apparently detached from this material during transport, before the sampling procedure in the laboratory (see Table 1). Another disadvantage of substrate sampling is that the fragile body may occasionally be damaged by excess shaking. Slide sampling seems to avoid this problem, providing placozoans without damage. The growth and reproductive patterns of placozoans on such slides with various other (mixed) organisms have been reported (Pearse, 1989). Slide sampling in this study also provided slides that could be used for rearing placozoans (data not shown). Therefore, a combination of both substrate and slide sampling seems to be the most effective way to study these microscopic animals from a local site.

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